Nucleophosmin/B23 activates Aurora A at the centrosome through phosphorylation of serine 89.

David Reboutier, Marie-Bérengère Troadec, Jean-Yves Cremet, Kenji Fukasawa, Claude Prigent

Published March 26, 2012

Nucleophosmin/B23 activates Aurora A at the centrosome through phosphorylation of serine 89

David Reboutier,[1,2] Marie-Bérengère Troadec,[1,2] Jean-Yves Cremet,[1,2] Kenji Fukasawa,[3] and Claude Prigent[1,2]

[1]Centre National de la Recherche Scientifique, Unité Mixte de Recherche UMR6290, 35043 Rennes, France
[2]Université de Rennes, Institut de Génétique et Développement de Rennes, Université Européenne de Bretagne, 35000 Rennes, France
[3]Molecular Oncology Program, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33613

Aurora A (AurA) is a major mitotic protein kinase involved in centrosome maturation and spindle assembly. Nucleophosmin/B23 (NPM) is a pleiotropic nucleolar protein involved in a variety of cellular processes including centrosome maturation. In the present study, we report that NPM is a strong activator of AurA kinase activity. NPM and AurA coimmunoprecipitate and colocalize to centrosomes in G2 phase, where AurA becomes active. In contrast with previously characterized AurA activators, NPM does not trigger autophosphorylation of AurA on threonine 288. NPM induces phosphorylation of AurA on serine 89, and this phosphorylation is necessary for activation of AurA. These data were confirmed in vivo, as depletion of NPM by ribonucleic acid interference eliminated phosphorylation of CDC25B on S353 at the centrosome, indicating a local loss of AurA activity. Our data demonstrate that NPM is a strong activator of AurA kinase activity at the centrosome and support a novel mechanism of activation for AurA.

Introduction

Mitosis is a complex process that allows the mother cell to divide into two daughter cells. During this event, equal segregation of genetic information is crucial. Indeed, any perturbation during chromosome segregation could lead to aneuploidy, a major cause of cancer (Ganem et al., 2009). The centrosome plays a major role in the cell cycle by serving as a signaling platform and by nucleating mitotic spindle microtubules. The centrosome cycle is regulated concomitantly with cell cycle progression and is controlled by several factors including the mitotic kinase Aurora A (AurA). AurA is a serine/threonine kinase that fulfills several key functions during the cell cycle. AurA is involved in G2/M transition, centrosome separation and maturation, mitotic spindle assembly, and in the G2/M and spindle assembly checkpoints. To fulfill these functions, AurA needs to be correctly located and activated at the appropriate time. Several AurA activators have been reported in the past decade, although the data are controversial. Indeed, the first reported AurA activators, Bora and Ajuba, have never been confirmed. Bora is rather an intermediate that stimulates PLK1 phosphorylation by AurA (Seki et al., 2008), and Ajuba has been demonstrated to not activate AurA in *Drosophila melanogaster* (Sabino et al., 2011). TPX2 (Eyers et al., 2003; Dodson and Bayliss, 2012), HEF1 (Pugacheva and Golemis, 2005), and, more recently, CEP192 (Joukov et al., 2010) and Arpc1b (Molli et al., 2010) have also been described as AurA activators. All these studies showed an increase in AurA phosphorylation on threonine 288 (T288), concomitant with activation of the kinase. T288 is located in the activation loop of the kinase and is directly involved in the activity of AurA. The fact that AurA is activated sequentially by several molecules suggests a fine tuning of the control of AurA activity and a complex regulatory network in which each activator increases the kinase activity for a specific function. It also opens new avenues to discover more activating proteins.

Nucleophosmin/B23 (NPM) is a phosphoprotein localized mainly in the nucleolus, where it exerts several of its functions. NPM also localizes to centrosomes, and a proportion of the protein continuously shuttles between the nucleus and the cytoplasm. NPM is involved in ribosome biogenesis, centrosome duplication, DNA repair, and response to stress. More recently, NPM was shown to be involved in mitotic spindle formation and regulation of microtubule spindle tension (Amin et al., 2008a,b). NPM has

D. Reboutier and M.-B. Troadec contributed equally to this paper.

Correspondence to Claude Prigent: claude.prigent@univ-rennes1.fr

Abbreviations used in this paper: IF, immunofluorescence; ShRNA, short hairpin RNA; WT, wild type.

The Rockefeller University Press $30.00
J. Cell Biol.
www.jcb.org/cgi/doi/10.1083/jcb.201107134

Published March 26, 2012

been implicated in the pathogenesis of several human malignancies and described both as an oncogene and a tumor suppressor, depending on the cell type and protein levels.

In the present study, we found that NPM is a strong activator of AurA in vitro. Our data show that NPM activates AurA through a novel mechanism that does not depend on T288 phosphorylation. Importantly, we showed that activation strongly depends on autophosphorylation of AurA on serine 89 (S89). We found that in vivo, AurA and NPM colocalize at the centrosome and coimmunoprecipitate. Activation of AurA by NPM was validated at the cellular level, as depletion of NPM by RNAi leads to a decrease in S353 phosphorylation in CDC25B, a target of AurA at the centrosome.

Results and discussion

We initially identified NPM in a screen designed to search for AurA substrates in mammalian cell extracts. We evaluated the activity of AurA in an in vitro kinase assay using GST-tagged H3 tail as a substrate (Scrittori et al., 2001). Purified histidine-tagged AurA was an active kinase, as it phosphorylated H3 (Fig. 1 A). We also confirmed that AurA phosphorylated GST-tagged NPM in vitro (Fig. 1 B, top). Moreover, we detected AurA autophosphorylation (Fig. 1, A and B). Surprisingly, AurA showed a stronger kinase activity against NPM than H3. An AurA concentration as low as 0.5 pmol was sufficient to phosphorylate NPM, whereas we did not detect any phosphorylation of H3 at that concentration of kinase (unpublished data). We only observed phosphorylation of H3 when the kinase concentration was increased up to 20 pmol (Fig. 1 A). AurA activity is commonly associated with phosphorylation of T288 on AurA (Bischoff et al., 1998). Intriguingly, we did not detect any increase in AurA T288 phosphorylation, as revealed by Western blotting using specific anti–phospho-T288 AurA antibody (Fig. 1 B, bottom).

Because AurA kinase activity was more intense when NPM was used as a substrate compared with H3, we investigated whether NPM could act as an activator of AurA kinase activity. We performed enzymatic assays with a limiting amount of kinase (0.5 pmol; Fig. 1 C). In the absence of NPM, autophosphorylation of AurA was not detectable, and phosphorylation of H3 was hardly detected (Fig. 1 C, first lane). The amount of AurA and H3 was kept unchanged, and we gradually increased the amount of NPM. As shown in Fig. 1 C (top, third to sixth lane), addition of increasing amounts of NPM resulted in increased phosphorylation of NPM as well as H3 and AurA. This clearly indicated that stimulation of AurA kinase activity correlated with the amount of NPM protein. Phosphorylation of H3 reached a plateau when the proportion of 5 pmol of NPM for 0.5 pmol of AurA was reached. During the past decade, several AurA activators were identified (Eyers et al., 2003; Pugacheva and Golemis, 2005; Hutterer et al., 2006; Joukov et al., 2010; Molli et al., 2010). However, mechanisms of activation have only been documented for TPX2 and CEP192. In both cases, activation was associated with a strong phosphorylation of AurA on T288. We observed no significant increase in T288 phosphorylation in AurA, as detected with an anti–phospho-T288 AurA antibody (Fig. 1 C), suggesting that NPM could activate AurA through an alternative mechanism.

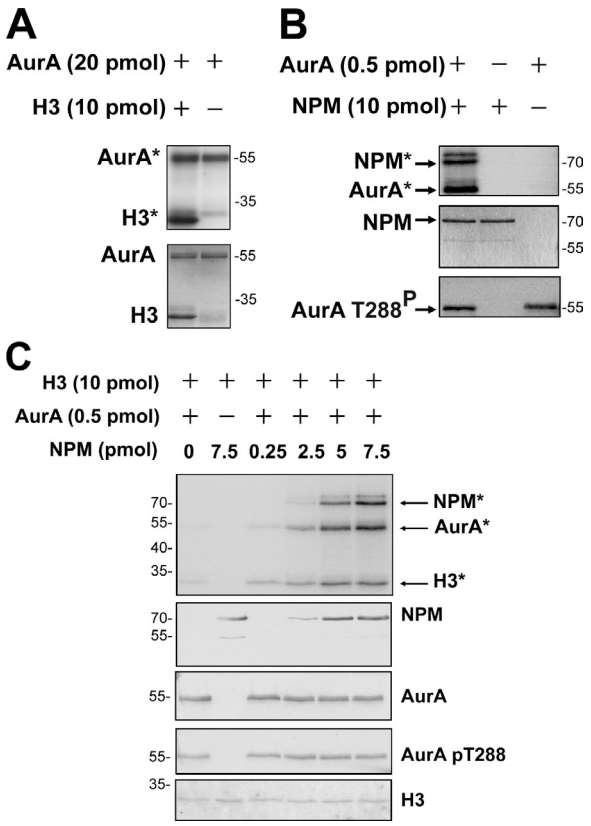

Figure 1. **AurA phosphorylates H3 and NPM in vitro.** (A) 20 pmol of AurA was incubated for 10 min at 37°C with 10 pmol of H3. H3 phosphorylation (*) was assessed by autoradiography (top), and the presence of AurA and H3 proteins was verified by Coomassie blue staining (bottom). (B) 0.5 pmol of AurA was incubated for 10 min at 37°C with 10 pmol of NPM. NPM phosphorylation was assessed by autoradiography (top), and the presence of NPM protein was assessed by Coomassie blue staining (middle). Autophosphorylation of AurA on T288 was verified by Western blotting (bottom). (C) 0.5 pmol of AurA was incubated with 10 pmol of H3 and the indicated amount of NPM. NPM, AurA, and H3 phosphorylation were assessed by autoradiography (top). NPM, H3, AurA, and AurA autophosphorylations on T288 (pT288) were evaluated by Western blotting or Coomassie blue staining. Molecular mass is indicated in kilodaltons.

TPX2 activates AurA through conformational changes and triggers protection of the T288 residue from dephosphorylation by PP1 phosphatase (Bayliss et al., 2003; Dodson and Bayliss, 2012). To confirm that NPM does not act like TPX2, we tested whether preincubation of AurA with increasing amounts of TPX2, or NPM, protected T288 phosphorylation. As previously reported, TPX2 protected AurA from dephosphorylation by PP1 (Fig. 2 A; Eyers et al., 2003). We repeated the experiment with NPM. Addition of increasing amounts of NPM to AurA did not protect T288 from being dephosphorylated by PP1 (Fig. 2 B). This finding indicates that NPM acts as an AurA kinase activator but, unlike TPX2, does not protect the T288 residue from being dephosphorylated. Even if T288 phosphorylation was not modified when AurA was activated by NPM, this phosphorylation event remained necessary for kinase activation. Indeed, NPM was not able to trigger AurA activation when T287/288 was mutated into alanine, a nonphosphorylable residue (Fig. S1). Any AurA protein detected by the anti–phospho-T288 antibody is usually considered as active, and this labeling is often used to measure

Published March 26, 2012

AurA activity (Saskova et al., 2008; Tong et al., 2008). However, three studies suggest that T288 phosphorylation is not an absolute readout to evaluate AurA activity. First, phosphatase inhibitor-2 can directly activate AurA without modifying T288 phosphorylation level (Satinover et al., 2004). Second, we showed that activity of *Xenopus laevis* AurA kinase phosphorylated on T295 (equivalent of T288 in human) could be down-regulated during oocyte maturation upon phosphorylation on S349 (Pascreau et al., 2008). Third, Dodson and Bayliss (2012) recently showed that binding of TPX2 without phosphorylation on T288 was sufficient to activate the kinase.

Fig. 1 C shows that AurA activation is accompanied by an increase in AurA autophosphorylation. However, Figs. 1 C and 2 B show that T288 is not increasingly phosphorylated in the presence of NPM. These results prompted us to think that phosphorylation of another residue could be involved in the activation. We used phosphoproteomics analysis to determine which residues were phosphorylated in AurA. We found that S89 was the only new residue on AurA to be phosphorylated in the presence of NPM (a result obtained from two independent proteomic facilities). S89 is located in the N terminus extension of AurA and has never previously been reported to be phosphorylated. This part of the kinase likely participates in localization and regulation of the activity of AurA (Rannou et al., 2008). This residue is well conserved among vertebrates. To assess whether S89 phosphorylation could play a role in AurA activation by NPM, we performed in vitro kinase assays with recombinant AurA proteins in which the residue S89 was mutated into an alanine (S89A, which cannot be phosphorylated) and into aspartic or glutamic acid (S89D or S89E, respectively, which should mimic a constitutive phosphorylation). We then compared the activity of the AurA S89 mutants with the wild-type (WT) kinase by monitoring H3 phosphorylation in the presence of increasing amounts of NPM. Fig. 2 C shows that S89A mutant was not activated by NPM anymore, thus indicating that S89 phosphorylation was necessary for activation of AurA by NPM. S89D or S89E mutants possess a basal activity similar to WT AurA and were not activated by NPM as S89A mutant. The fact that these mutants did not behave like phosphomimetic mutants was not surprising, and it is now well accepted that glutamic acid or aspartic acid does not systematically mimic phosphorylation (Eyers and Maller, 2004; Pascreau et al., 2008; Anthis et al., 2009). It is likely that, similar to the results obtained by Paleologou et al. (2008), mutation of S89 into glutamic or aspartic acid does not exactly modify AurA conformation as phosphorylation of S89 does. We then searched for the NPM domain responsible for AurA activation. We used four constructs to produce various truncated NPM proteins (Fig. 2 D). Fig. 2 E shows that the domain that allowed activation of AurA was in the C terminus of NPM in the P3 fragment. To date, no specific function has been attributed to this NPM domain.

To determine the in vivo relevance of the NPM and AurA interaction, we investigated whether the two proteins could colocalize in vivo. NPM is a nucleolar protein, also reported to localize to centrosomes (Zatsepina et al., 1995), between centrioles (Okuda et al., 2000). Similarly, AurA localizes to centrosomes from the end of S phase to the beginning of G1 phase

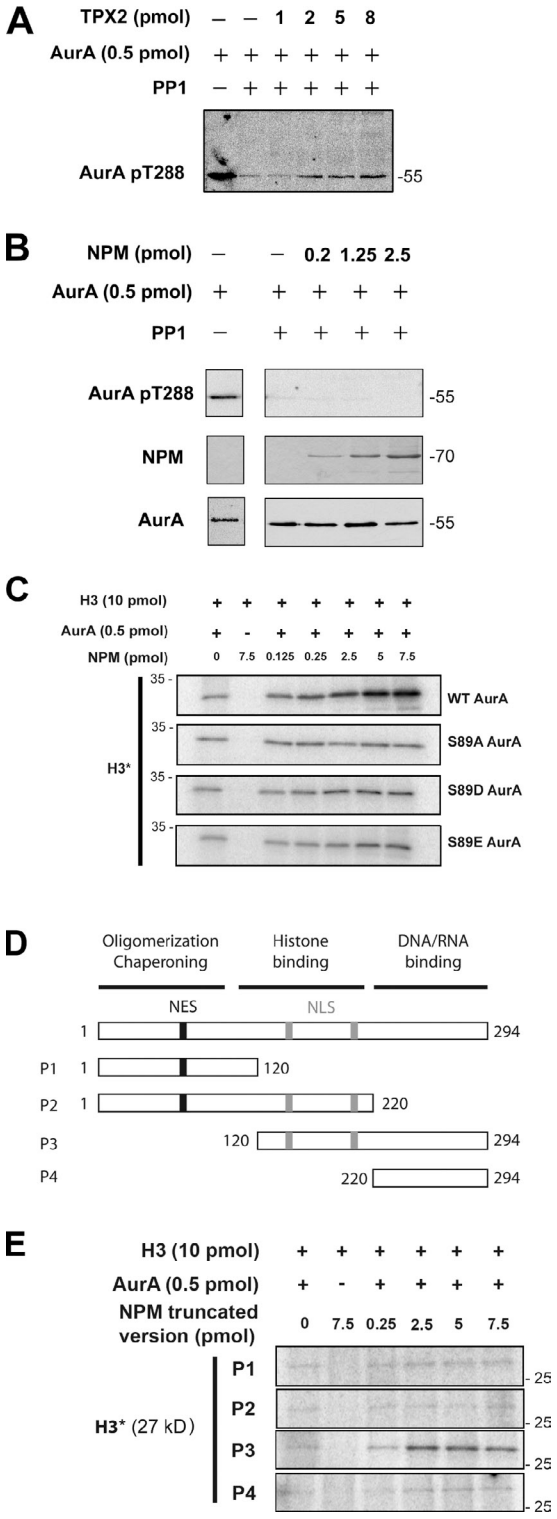

Figure 2. **NPM activates AurA through S89 phosphorylation without protecting T288 from dephosphorylation.** (A and B) 0.5 pmol of AurA was incubated with the indicated amount of TPX2 or NPM and then treated with 0.05 U of PP1. AurA, NPM protein, and AurA autophosphorylation on T288 (pT288) were evaluated by Western blotting. (C) 0.5 pmol of WT AurA or the indicated mutant was incubated with 10 pmol of H3 and the indicated amount of NPM. H3 phosphorylation (*) was quantified by autoradiography. (D) A scheme representing the different fragments of NPM used in E. NES, nuclear export signal. (E) 0.5 pmol of AurA was incubated with 10 pmol of H3 and the indicated amounts of the fragments of NPM presented in D. H3 phosphorylation was evaluated by autoradiography. Molecular mass is indicated in kilodaltons.

Published March 26, 2012

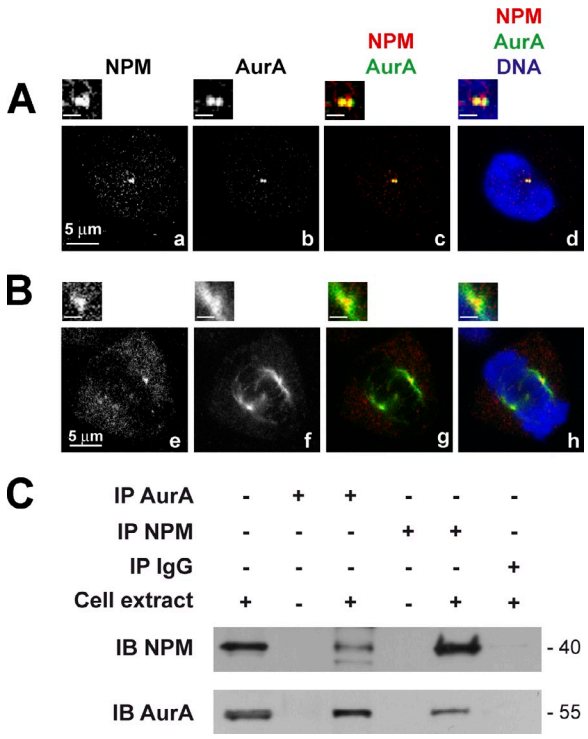

Figure 3. **NPM and AurA colocalize from the end of G2 to M phase and interact in vivo.** (A and B) IF microscopy of cells in G2 phase (A) or metaphase (B). (insets) Enlargements of the centrosome area. Bars, 1 µm. (C) A Western blot showing coimmunoprecipitation (IP) of AurA and NPM from HeLa cell protein extract. Molecular mass is indicated in kilodaltons.

(Gopalan et al., 1997; Roghi et al., 1998). We examined the localization of both proteins by confocal microscopy using a rabbit polyclonal anti-NPM antibody and a mouse monoclonal anti-AurA antibody. As expected, both proteins were found at the centrosome at the end of interphase as well as in mitosis (Fig. 3, A and B). To study the in vivo physical interaction of NPM and AurA, we immunoprecipitated each protein individually from HeLa cells and used Western blotting to detect the presence of the other protein in the immunoprecipitate. Immunoprecipitations were performed with nonsynchronized cells as well as with cells synchronized in mitosis. We obtained the same result in both cases: NPM was detected in AurA immunoprecipitate, and AurA was detected in NPM immunoprecipitate (Fig. 3 C). Therefore, both proteins not only colocalize but also interact in vivo.

To investigate whether NPM has any effect on AurA in vivo, we examined the phosphorylation of two well-characterized substrates of AurA at the centrosome. First, we monitored PLK1 phosphorylation. This kinase is phosphorylated by AurA on T210, and this event is necessary for mitotic entry (Macůrek et al., 2008; Seki et al., 2008). We obtained >80% of NPM depletion by transfecting U2OS cells with a plasmid coding a short hairpin RNA (ShRNA; Fig. S2 A). PLK1 expression level and phosphorylation on T210 were first quantified by Western blotting. Fig. S2 A indicates that they were not affected by NPM depletion. The lack of modification in PLK1 phosphorylation status was confirmed by immunofluorescence (IF), as PLK1 phospho-T210 centrosomal labeling was not affected in NPM-depleted cells (Fig. S2 B). Next, we examined the effect of NPM depletion on CDC25B phosphorylation. CDC25B is phosphorylated on serine 353 by AurA at the end of G2 just before G2/M transition. This phosphorylation event can be monitored using the phospho-specific SE96 antibody (Dutertre et al., 2004; Cazales et al., 2005; Krystyniak et al., 2006). Under these conditions, we analyzed the phosphorylation status of CDC25B on S353 using Western blotting. We detected a strong decrease in phosphorylation of S353 in CDC25B when NPM was depleted, though the protein levels of CDC25B and AurA were unchanged (Fig. 4 A). We obtained the same results by using two other ShRNAs targeting different sequences (Fig. S3). In contrast to NPM depletion, NPM overexpression (with NPM tagged at the N or C terminus end) did not change the phosphorylation of S353 in CDC25B, suggesting that under physiological conditions, AurA is likely to be fully activated by NPM (unpublished data). We further asked in vitro whether NPM was able to activate phosphorylation of CDC25B by AurA, similar to what we observed concerning H3. Fig. 4 B confirmed that addition of increasing amounts of NPM activated the kinase and increased phosphorylation of CDC25B. To verify that lack of S353 phosphorylation of CDC25B in the absence of NPM was occurring at the centrosome, we used the same anti–phospho-CDC25B antibody to evaluate the phosphorylation of S353 in proliferating cells by IF. Late G2 cells were distributed according to the intensity of centrosomal labeling. In control cells, ∼10% of cells showed no labeling, 45% showed weak labeling, and ∼45% of cells showed strong labeling (Fig. 4 C). When NPM was depleted, 35% of cells showed no labeling, 50% showed weak labeling, and only 15% of cells showed strong labeling (Fig. 4 D). These results clearly indicated a decrease in CDC25B phosphorylation on S353 in the absence of NPM, demonstrating that AurA kinase was less active at the centrosome in the absence of NPM. They also indicated that activation of AurA by NPM was quite specific toward CDC25B, as PLK1 phosphorylation was not affected by NPM depletion. This last result could also be explained by the fact that phosphorylation of PLK1 by AurA is already stimulated by Bora (Macůrek et al., 2008; Seki et al., 2008). Importantly, similar to our in vitro findings, phosphorylation of AurA on T288 was not dependent on the presence of NPM (Fig. 4 E).

We then wanted to confirm in vivo that AurA activation by NPM acts through phosphorylation of S89 on AurA. Because of lack of S89 phospho-specific antibody, we could not directly evaluate the phosphorylation state of S89 in the presence or absence of NPM. To study the effect of S89 phosphorylation on AurA activity in vivo, we examined the effect of expression of S89 mutant versions of AurA on CDC25B phospho-S353 labeling at the centrosome. Cells were first transfected with plasmids encoding for WT, S89A, S89D, or S89E GFP-tagged AurA proteins, resulting in physiological level of expression of the WT or mutated kinases. Endogenous AurA was depleted by transfection with an siRNA that does not target the transfected versions of the kinase. Fig. 5 A indicates that, similar to NPM depletion, AurA depletion triggered an increase in the number of cells with centrosomes that showed no CDC25B phospho-S353 labeling and a decrease in the number of cells presenting a strong CDC25B phospho-S353 labeling. This phenotype was rescued by WT AurA but not by S89A AurA, thus suggesting that phosphorylation of AurA on S89 is necessary for the kinase

Published March 26, 2012

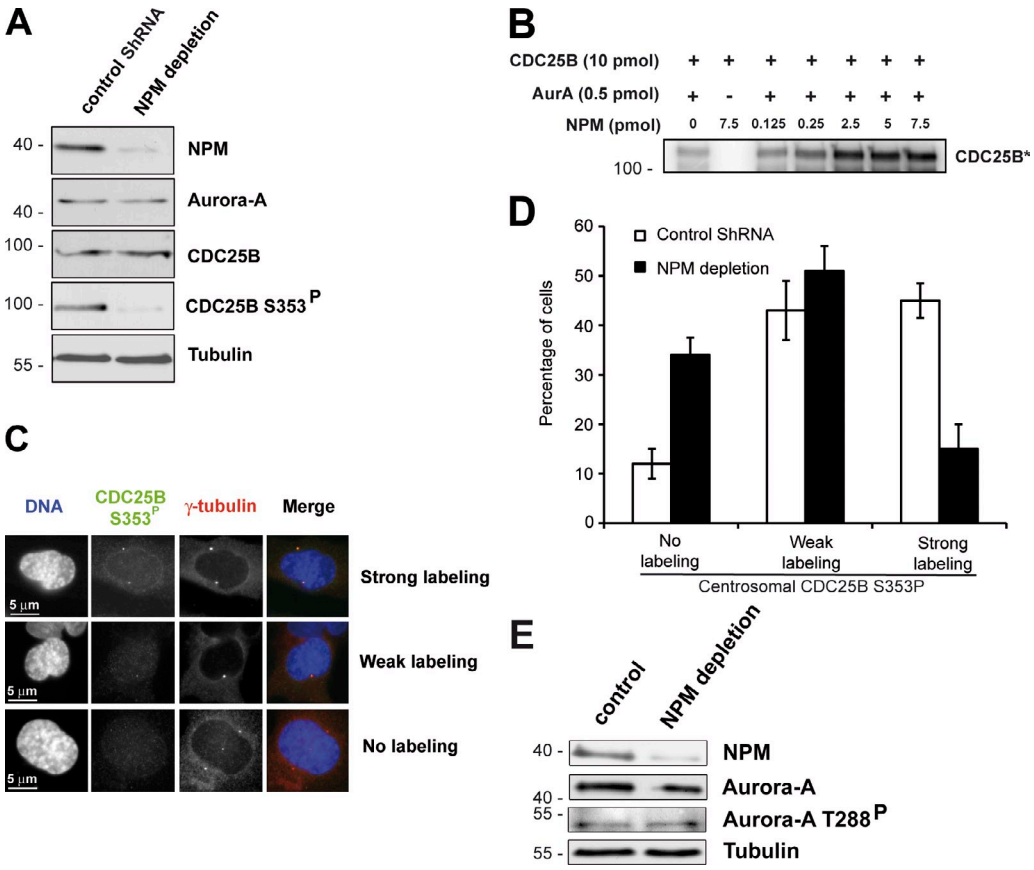

Figure 4. **NPM depletion triggers a decrease in CDC25B phosphorylation.** (A) Protein lysates prepared from control or NPM-depleted U2OS cells were immunoblotted with the indicated antibodies. (B) 0.5 pmol of AurA was incubated with 10 pmol of CDC25B and the indicated amount of NPM. CDC25B phosphorylation (*) was quantified by autoradiography. (C) IF characterization of CDC25B phospho-S353 labeling in control or NPM-depleted U2OS cells. Control or NPM-depleted cells were classified into three categories (no, weak, or strong labeling) according to the intensity of CDC25B phospho-S353 labeling. (D) A histogram representing the distribution of control or NPM-depleted cells in each category. Values are means of three independent experiments. 50 cells were counted for each experiment. Error bars represent SEM. (E) Protein lysates prepared from control or NPM-depleted U2OS cells were immunoblotted with anti-NPM, anti-AurA, anti–AurA phospho-T288, and with anti–β-tubulin as a loading control. Molecular mass is indicated in kilodaltons.

to be activated by NPM. In agreement with our previous biochemical data showing that S89D and S89E mutations were not phosphomimetic, S89D and S89E mutants could not rescue AurA depletion.

AurA plays many crucial roles in cell cycle regulation and mitosis achievement. Notably, AurA was proven to be involved in microtubule growth through recruitment of microtubule-nucleating factors at the centrosomes (Joukov, 2011). NPM also plays key roles in centrosome biology. It has recently been shown to be involved in mitotic spindle formation and regulation of spindle microtubule nucleation and tension (Amin et al., 2008a,b). NPM involvement in microtubule dynamics could act through its interaction with AurA. To test this hypothesis, we examined the effect of NPM or AurA depletion on microtubule nucleation. Cells were treated by 33 μM nocodazole for 3 h to depolymerize microtubules and were then washed four times with prewarmed fresh medium. 2 min after washes, cells were fixed and labeled for α- and γ-tubulin, and then microtubules nucleating from centrosomes in G2-phase cells were measured. Fig. 5 B showed that microtubules were shorter in cells depleted for NPM or AurA than in control. WT AurA was able to rescue this phenotype, contrary to S89A AurA. This suggested that phosphorylation of AurA on S89 is involved in microtubule nucleation and that NPM likely participates in nucleation through activation of AurA. It is particularly interesting to notice that CDC25B, like NPM, is also involved in centrosome duplication (Boutros et al., 2007) and mitotic spindle formation through microtubule nucleation (Gabrielli et al., 1996). Thus, our study suggests a connection between two independent pathways implicating (a) cyclin E/Cdk2, NPM, or ROCK II and (b) AurA, CDC25B, or Centrin 2 and could open up new perspectives concerning centrosome duplication and maturation studies.

Materials and methods

DNA constructs and protein purification

pGEX-B23 was provided by K. Fukasawa. pGEX-4T3-NPM-P1, -P2, -P3, and -P4 were gifts from I. Hoffmann (German Cancer Research Center, Heidelberg, Germany). pMAL-C2-CDC25B3 was a gift from B. Ducommun (Institut des Technologies Avancées en sciences du Vivant, Toulouse, France). AurA and H3 tail cDNA were cloned in pET29 and pGEX vectors, respectively. AurA mutants (S89A, S89D, S89E, and T287/288A) were generated with the QuikChange mutagenesis kit (Agilent Technologies). AurA depletion in U2OS cells was achieved as described in Dutertre et al. (2004). NPM depletion in U2OS cells was achieved after transfection with pLKO plasmids (available from GenBank/EMBL/DDBJ under accession no. NM_002520; Sigma-Aldrich) targeting the following sequences: no. 1,

Published March 26, 2012

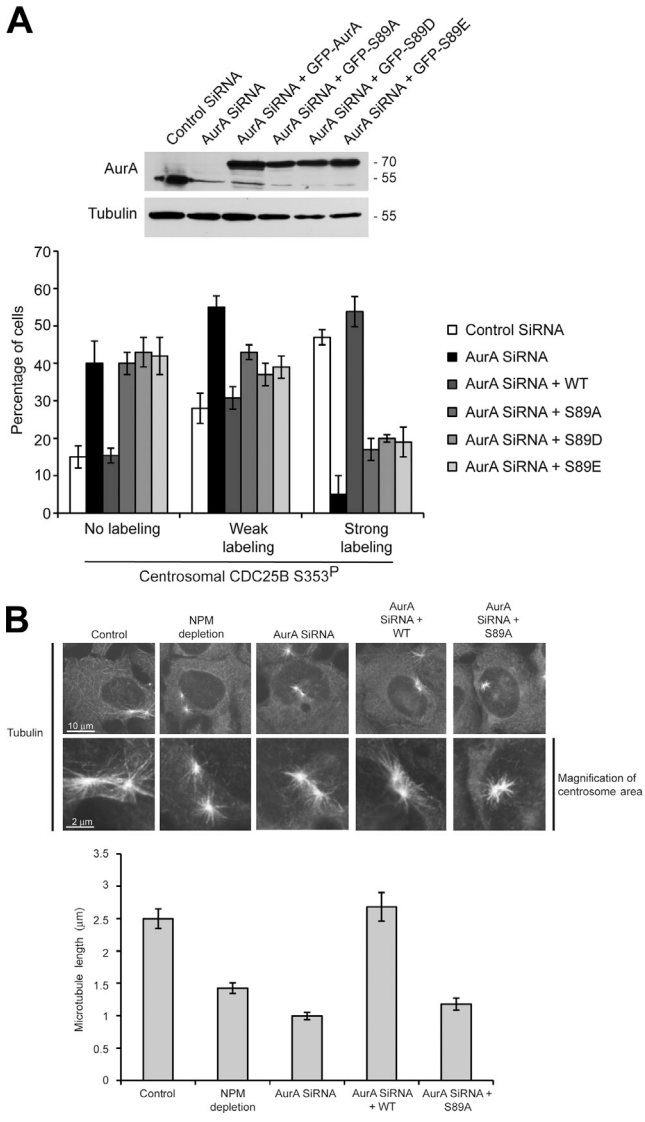

Figure 5. **AurA S89 phosphorylation is required for CDC25B phosphorylation and microtubule nucleation at centrosomes.** (A) Expression of AurA in U2OS cells transfected with the indicated siRNA and/or plasmids was verified by Western blotting (top). Anti–β-tubulin serves as a loading control. For each corresponding cell population, the intensity of centrosomal CDC25B phospho-S353 labeling was evaluated, and cells were classified into three categories (no, weak, or strong labeling). Data are summarized in the histogram (bottom). Values are means of three independent experiments. 50 cells were counted for each experiment. Error bars represent SEM. (B) IF microscopy of cells with nucleating microtubules at centrosomes. The length of microtubules is summarized in the histogram. Data are representative of three independent experiments. Error bars represent SEM.

5′-CCGGGCCGACAAAGATTATCACTTTCTCGAGAAAGTGATAATCTTTGTCGGCTTT-3′; no. 2, 5′-CCGGGCAAAGGATGAGTTGCACATTCTCGAGAATGTGCAACTCATCCTTTGCTTTTTG-3′; and no. 3, 5′-CCGGGCCTAGTTCTGTAGAAGACATTCTCGAGAATGTCTTCTACAGAACTAGGTTTTTG-3′. Luciferase ShRNA was used as a control. AurA WT and S89 mutant versions were cloned in a pEGFP-C1 vector with the AurA promoter sequence to allow physiological expression level. For recombinant protein production, BL21 *Escherichia coli* were transformed with plasmids, induced by 1 mM IPTG for 4 h, pelleted, and frozen at −80°C. Purification of histidine-tagged proteins (human AurA and histone H3) was performed as previously described (Roghi et al., 1998). In brief, bacterial pellets were lysed and loaded onto Ni–nitrilotriacetic acid agarose beads (QIAGEN) and washed, and proteins were eluted with buffer containing imidazole. Purification of GST-tagged proteins (human NPM and NPM fragments) was performed with glutathione Sepharose 4B (GE Healthcare) in accordance with the manufacturer's instructions. Pellets were lysed and loaded into glutathione Sepharose 4B. MBP-CDC25B3 was produced in JM109 bacteria and affinity purified on amylose beads following the manufacturer's instructions (New England Biolabs, Inc.). Purified proteins were stored at −80°C or in 50% glycerol at −20°C.

Cell lines and transfection

Human HeLa cells were grown in DME with penicillin and streptomycin (Invitrogen) and 10% FCS (GE Healthcare). Human U2O2 cells were grown in McCoy's Medium with penicillin and streptomycin (Invitrogen) and 10% FCS. Cells were transfected in culture medium using jetPRIME (Polyplus Transfection) in accordance with the manufacturer's instructions. Media were changed after 24 h. Mitotic cells were collected by mitotic shake-off after incubation in 1 µg/ml taxol for 16 h. For expression of GFP-AurA WT or S89 mutant versions, cells were transfected for 24 h with the appropriate plasmid, and endogenous AurA was then depleted by transfection with siRNA for 24 h.

IF

Cells were preliminarily plated onto glass coverslips. The cells were then washed in cold PBS with calcium, fixed with methanol at −20°C for 5 min (3 min for CDC25B phospho-S353 and CDC25B), and then washed three times in cold PBS and saturated with 5% PBS-BSA (5% PBS-SVF for CDC25B phospho-S353 and CDC25B) for 1 h at room temperature. Antibodies in 5% PBS-BSA (5% PBS-SVF for CDC25B phospho-S353 and CDC25B) were added onto the following cells: rabbit anti–NPM PabN1 (1:100; Shinmura et al., 2005); mouse anti–γ-tubulin (1:500, clone GTU88; Sigma-Aldrich); mouse anti-AurA (clone 35C11:20; Cremet et al., 2003); CDC25B and CDC25B phospho-S353 (1:100; a gift from B. Ducommun); PLK1 (Invitrogen); and PLK1 phospho-T210 (1:1,000; BD). These were left overnight at 4°C and then washed three times and incubated in the dark with secondary antibodies (anti–mouse and anti–rabbit Alexa Fluor 488 or 555, 1:1,000; Invitrogen) for 1 h at room temperature. After three final washes with PBS, coverslips were mounted with Prolong gold (Invitrogen) with 1 µg/ml DAPI (Sigma-Aldrich). Cells were examined using a fluorescent microscope (DMRXA2; Leica) with a 63× oil immersion objective. Images were processed using MetaMorph software (Universal Imaging). Cells were also observed with a confocal microscope (SP2; Leica), and images were processed with Leica software.

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay buffer containing antiprotease (Complete; Roche), and lysates were clarified by centrifugation (13,000 rpm for 30 min at 4°C). Proteins were assayed by Bradford assay (Bio-Rad Laboratories). Equal amounts of protein lysates in Laemmli were loaded onto 12.5% SDS-PAGE gel for electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with TBST–4% milk (5% BSA for NPM) and then incubated overnight with the following antibodies: mouse anti–β-tubulin (1:2,000); mouse anti-NPM (1:2,000; Sigma-Aldrich); mouse anti-AurA (clone 35C1, 1:100; Cremet et al., 2003); rabbit anti-CDC25B and CDC25B phospho-S353 (1:1,000; a gift from B. Ducommun); mouse anti-PLK1 (Invitrogen); and PLK1 phospho-T210. Secondary antibodies coupled with HRP (Jackson ImmunoResearch Laboratories, Inc.) were incubated for 1 h, and antibody binding was detected by ECL (Pico or Dura; Thermo Fisher Scientific).

In vitro kinase assays

Different amounts of recombinant AurA, recombinant nucleophosmin, and recombinant H3 or CDC25B—as stated in the figures—were mixed in 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM DTT, 0.01% Triton X-100, and 10 mM $MgCl_2$. Then, 100 µM ATP and 2 µCi γ-[^{32}P]ATP were added to the reaction mixture and incubated at 37°C for 10 min. Reactions were stopped in Laemmli buffer, boiled for 5 min, and resolved on a 12.5% SDS-PAGE gel. Gels were either stained with Coomassie blue, fixed, dried, and autoradiographied with Storm 840 (Molecular Dynamics) or were transferred onto nitrocellulose membrane, autoradiographied for 3 d on X-OMAT films (Kodak), and then blocked with PBS–4% milk and subjected to immunoblotting.

Mass spectrometry analysis

AurA was proven to be phosphorylated even when purified from *E. coli* as a recombinant protein (Haydon et al., 2003). To clearly discriminate between phosphorylation events, 1 µg of recombinant AurA was incubated for 20 min at 37°C either alone or in presence of 20 mM ATP or in presence of 20 mM ATP and 1 µg NPM. Enzymatic reactions were stopped with

Laemmli buffer. Proteins were separated in SDS-PAGE, and the gel was stained with Coomassie blue.

The gel slices were destained with 50% methanol and then reduced in 10 mM DTT for 1 h at 56°C and alkylated in 55 mM chloroacetamide for 1 h at room temperature. After washing in 50 mM ammonium bicarbonate, the gel pieces were shrunk in 100% acetonitrile. The digestion was performed using trypsin in 50 mM ammonium bicarbonate for 8 h at 37°C and 600 rpm. The peptides were finally extracted in 90% acetonitrile/0.5 M Urea and dried in SpeedVac (Thermo Fisher Scientific). Samples were resolubilized in 5% acetonitrile and 0.2% formic acid. Samples were separated on a homemade C18 column (150 µm × 10 cm) using a nanoLC 2D system (Eksigent). A 56-min gradient from 5–60% acetonitrile (0.2% formic acid) was used to elute peptides from a homemade reversed-phase column (150 µm i.d. × 100 mm) with a flow rate set at 600 nanoliters/min.

The column was directly connected to a nanoprobe interfaced with a mass spectrometer (LTQ Orbitrap Velos; Thermo Fisher Scientific). Each full mass spectrometry spectrum was followed by 12 tandem mass spectrometry (MS/MS) spectra (13 scan events), in which the 12 most abundant multiply charged ions were selected for MS/MS sequencing. MS/MS experiments were performed using collision-induced dissociation in the linear ion trap. The data were processed using the Mascot 2.1 search engine (Matrix Science) with tolerance parameters set to 15 parts per million and 0.5 D for the precursor and the fragment ions, respectively. The selected variable modifications were carbamidomethyl (C), deamidation (NQ), oxidation (M), and phosphorylation (STY). The selected database was Human International Protein Index database (v.3.54) with 150,858 sequences.

Immunoprecipitation

Protein G Sepharose Fast Flow (GE Healthcare) and monoclonal anti-AurA (Cremet et al., 2003) clone 35C1 or monoclonal anti-B23 (Sigma-Aldrich) were incubated overnight at 4°C and then washed extensively. Cells were lysed in 20 mM EDTA, 15 mM $MgCl_2$, 20 mM Hepes, pH 7.5, 0.1% Triton X-100, antiprotease (Complete), and antiphosphatase cocktail (50 mM NaF, 80 mM β-glycerophosphate, and 1 mM Na_3VO_4). Cell lysates were loaded onto Protein G Sepharose Fast Flow overnight at 4°C and then subjected to extensive washes in lysis buffer for 4 h at 4°C. Samples were finally eluted in Laemmli and boiled for 5 min at 90°C or used in a kinase assay.

Microtubule nucleation assay

Cells were incubated for 3 h with 33 µM nocodazole in culture medium. Then, the medium was removed, and cells were washed four times with fresh prewarmed medium. 2 min after the last wash, cells were fixed with cold methanol and then immunolabeled for γ- and β-tubulin. The length of microtubules was measured with MetaMorph software.

Online supplemental material

Fig. S1 indicates that NPM was not able to activate a T287/288A AurA mutant. Fig. S2 shows that NPM depletion did not affect phosphorylation of PLK1. Fig. S3 shows that three different ShRNAs designed to target NPM triggered a decrease in CDC25B phosphorylation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201107134/DC1.

We thank Jean-Philippe Gagné (Plate-forme de Protéomique, Centre de Génomique de Québec, Québec, Canada) and Mélanie Lagarrigue (Plate-forme Protéomique Biogenouest, Rennes, France) for proteomics analysis. The SE96 antibody was provided by B. Ducommun, and NPM constructs were provided by I. Hoffmann.

This work was supported by the Centre National de la Recherche Scientifique, Université de Rennes 1, Institut National du Cancer, Ligue Nationale Contre le Cancer (Equipe Labelisée), and Agence Nationale de la Recherche.

Submitted: 31 July 2011
Accepted: 28 February 2012

References

Amin, M.A., S. Matsunaga, S. Uchiyama, and K. Fukui. 2008a. Depletion of nucleophosmin leads to distortion of nucleolar and nuclear structures in HeLa cells. *Biochem. J.* 415:345–351. http://dx.doi.org/10.1042/BJ20081411

Amin, M.A., S. Matsunaga, S. Uchiyama, and K. Fukui. 2008b. Nucleophosmin is required for chromosome congression, proper mitotic spindle formation, and kinetochore-microtubule attachment in HeLa cells. *FEBS Lett.* 582:3839–3844. http://dx.doi.org/10.1016/j.febslet.2008.10.023

Anthis, N.J., J.R. Haling, C.L. Oxley, M. Memo, K.L. Wegener, C.J. Lim, M.H. Ginsberg, and I.D. Campbell. 2009. Beta integrin tyrosine phosphorylation is a conserved mechanism for regulating talin-induced integrin activation. *J. Biol. Chem.* 284:36700–36710. http://dx.doi.org/10.1074/jbc.M109.061275

Bayliss, R., T. Sardon, I. Vernos, and E. Conti. 2003. Structural basis of Aurora-A activation by TPX2 at the mitotic spindle. *Mol. Cell.* 12:851–862. http://dx.doi.org/10.1016/S1097-2765(03)00392-7

Bischoff, J.R., L. Anderson, Y. Zhu, K. Mossie, L. Ng, B. Souza, B. Schryver, P. Flanagan, F. Clairvoyant, C. Ginther, et al. 1998. A homologue of *Drosophila* aurora kinase is oncogenic and amplified in human colorectal cancers. *EMBO J.* 17:3052–3065. http://dx.doi.org/10.1093/emboj/17.11.3052

Boutros, R., V. Lobjois, and B. Ducommun. 2007. CDC25B involvement in the centrosome duplication cycle and in microtubule nucleation. *Cancer Res.* 67:11557–11564. http://dx.doi.org/10.1158/0008-5472.CAN-07-2415

Cazales, M., E. Schmitt, E. Montembault, C. Dozier, C. Prigent, and B. Ducommun. 2005. CDC25B phosphorylation by Aurora-A occurs at the G2/M transition and is inhibited by DNA damage. *Cell Cycle*. 4:1233–1238. http://dx.doi.org/10.4161/cc.4.9.1964

Cremet, J.Y., S. Descamps, F. Vérite, A. Martin, and C. Prigent. 2003. Preparation and characterization of a human aurora-A kinase monoclonal antibody. *Mol. Cell. Biochem.* 243:123–131. http://dx.doi.org/10.1023/A:1021608012253

Dodson, C.A., and R. Bayliss. 2012. Activation of Aurora-A kinase by protein partner binding and phosphorylation are independent and synergistic. *J. Biol. Chem.* 287:1150–1157. http://dx.doi.org/10.1074/jbc.M111.312090

Dutertre, S., M. Cazales, M. Quaranta, C. Froment, V. Trabut, C. Dozier, G. Mirey, J.P. Bouché, N. Theis-Febvre, E. Schmitt, et al. 2004. Phosphorylation of CDC25B by Aurora-A at the centrosome contributes to the G2-M transition. *J. Cell Sci.* 117:2523–2531. http://dx.doi.org/10.1242/jcs.01108

Eyers, P.A., and J.L. Maller. 2004. Regulation of *Xenopus* Aurora A activation by TPX2. *J. Biol. Chem.* 279:9008–9015. http://dx.doi.org/10.1074/jbc.M312424200

Eyers, P.A., E. Erikson, L.G. Chen, and J.L. Maller. 2003. A novel mechanism for activation of the protein kinase Aurora A. *Curr. Biol.* 13:691–697. http://dx.doi.org/10.1016/S0960-9822(03)00166-0

Gabrielli, B.G., C.P. De Souza, I.D. Tonks, J.M. Clark, N.K. Hayward, and K.A. Ellem. 1996. Cytoplasmic accumulation of cdc25B phosphatase in mitosis triggers centrosomal microtubule nucleation in HeLa cells. *J. Cell Sci.* 109:1081–1093.

Ganem, N.J., S.A. Godinho, and D. Pellman. 2009. A mechanism linking extra centrosomes to chromosomal instability. *Nature*. 460:278–282. http://dx.doi.org/10.1038/nature08136

Gopalan, G., C.S. Chan, and P.J. Donovan. 1997. A novel mammalian, mitotic spindle–associated kinase is related to yeast and fly chromosome segregation regulators. *J. Cell Biol.* 138:643–656. http://dx.doi.org/10.1083/jcb.138.3.643

Haydon, C.E., P.A. Eyers, L.D. Aveline-Wolf, K.A. Resing, J.L. Maller, and N.G. Ahn. 2003. Identification of novel phosphorylation sites on *Xenopus laevis* Aurora A and analysis of phosphopeptide enrichment by immobilized metal-affinity chromatography. *Mol. Cell. Proteomics*. 2:1055–1067. http://dx.doi.org/10.1074/mcp.M300054-MCP200

Hutterer, A., D. Berdnik, F. Wirtz-Peitz, M. Zigman, A. Schleiffer, and J.A. Knoblich. 2006. Mitotic activation of the kinase Aurora-A requires its binding partner Bora. *Dev. Cell*. 11:147–157. http://dx.doi.org/10.1016/j.devcel.2006.06.002

Joukov, V. 2011. Aurora kinases and spindle assembly: Variations on a common theme? *Cell Cycle*. 10:895–903. http://dx.doi.org/10.4161/cc.10.6.14909

Joukov, V., A. De Nicolo, A. Rodriguez, J.C. Walter, and D.M. Livingston. 2010. Centrosomal protein of 192 kDa (Cep192) promotes centrosome-driven spindle assembly by engaging in organelle-specific Aurora A activation. *Proc. Natl. Acad. Sci. USA*. 107:21022–21027. http://dx.doi.org/10.1073/pnas.1014664107

Krystyniak, A., C. Garcia-Echeverria, C. Prigent, and S. Ferrari. 2006. Inhibition of Aurora A in response to DNA damage. *Oncogene*. 25:338–348.

Macůrek, L., A. Lindqvist, D. Lim, M.A. Lampson, R. Klompmaker, R. Freire, C. Clouin, S.S. Taylor, M.B. Yaffe, and R.H. Medema. 2008. Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. *Nature*. 455:119–123. http://dx.doi.org/10.1038/nature07185

Molli, P.R., D.Q. Li, R. Bagheri-Yarmand, S.B. Pakala, H. Katayama, S. Sen, J. Iyer, J. Chernoff, M.Y. Tsai, S.S. Nair, and R. Kumar. 2010. Arpc1b, a centrosomal protein, is both an activator and substrate of Aurora A. *J. Cell Biol.* 190:101–114. http://dx.doi.org/10.1083/jcb.200908050

Okuda, M., H.F. Horn, P. Tarapore, Y. Tokuyama, A.G. Smulian, P.K. Chan, E.S. Knudsen, I.A. Hofmann, J.D. Snyder, K.E. Bove, and K. Fukasawa. 2000. Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. *Cell*. 103:127–140. http://dx.doi.org/10.1016/S0092-8674(00)00093-3

Paleologou, K.E., A.W. Schmid, C.C. Rospigliosi, H.Y. Kim, G.R. Lamberto, R.A. Fredenburg, P.T. Lansbury Jr., C.O. Fernandez, D. Eliezer, M. Zweckstetter,

and H.A. Lashuel. 2008. Phosphorylation at Ser-129 but not the phosphomimics S129E/D inhibits the fibrillation of alpha-synuclein. *J. Biol. Chem.* 283:16895–16905. http://dx.doi.org/10.1074/jbc.M800747200

Pascreau, G., J.G. Delcros, N. Morin, C. Prigent, and Y. Arlot-Bonnemains. 2008. Aurora-A kinase Ser349 phosphorylation is required during *Xenopus laevis* oocyte maturation. *Dev. Biol.* 317:523–530. http://dx.doi.org/10.1016/j.ydbio.2008.02.053

Pugacheva, E.N., and E.A. Golemis. 2005. The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome. *Nat. Cell Biol.* 7:937–946. http://dx.doi.org/10.1038/ncb1309

Rannou, Y., M.B. Troadec, C. Petretti, F. Hans, S. Dutertre, S. Dimitrov, and C. Prigent. 2008. Localization of aurora A and aurora B kinases during interphase: Role of the N-terminal domain. *Cell Cycle*. 7:3012–3020. http://dx.doi.org/10.4161/cc.7.19.6718

Roghi, C., R. Giet, R. Uzbekov, N. Morin, I. Chartrain, R. Le Guellec, A. Couturier, M. Dorée, M. Philippe, and C. Prigent. 1998. The *Xenopus* protein kinase pEg2 associates with the centrosome in a cell cycle-dependent manner, binds to the spindle microtubules and is involved in bipolar mitotic spindle assembly. *J. Cell Sci.* 111:557–572.

Sabino, D., N.H. Brown, and R. Basto. 2011. *Drosophila* Ajuba is not an Aurora-A activator but is required to maintain Aurora-A at the centrosome. *J. Cell Sci.* 124:1156–1166. http://dx.doi.org/10.1242/jcs.076711

Saskova, A., P. Solc, V. Baran, M. Kubelka, R.M. Schultz, and J. Motlik. 2008. Aurora kinase A controls meiosis I progression in mouse oocytes. *Cell Cycle*. 7:2368–2376.

Satinover, D.L., C.A. Leach, P.T. Stukenberg, and D.L. Brautigan. 2004. Activation of Aurora-A kinase by protein phosphatase inhibitor-2, a bifunctional signaling protein. *Proc. Natl. Acad. Sci. USA*. 101:8625–8630. http://dx.doi.org/10.1073/pnas.0402966101

Scrittori, L., F. Hans, D. Angelov, M. Charra, C. Prigent, and S. Dimitrov. 2001. pEg2 aurora-A kinase, histone H3 phosphorylation, and chromosome assembly in *Xenopus* egg extract. *J. Biol. Chem.* 276:30002–30010. http://dx.doi.org/10.1074/jbc.M102701200

Seki, A., J.A. Coppinger, C.Y. Jang, J.R. Yates, and G. Fang. 2008. Bora and the kinase Aurora a cooperatively activate the kinase Plk1 and control mitotic entry. *Science*. 320:1655–1658. http://dx.doi.org/10.1126/science.1157425

Shinmura, K., P. Tarapore, Y. Tokuyama, K.R. George, and K. Fukasawa. 2005. Characterization of centrosomal association of nucleophosmin/B23 linked to Crm1 activity. *FEBS Lett.* 579:6621–6634. http://dx.doi.org/10.1016/j.febslet.2005.10.057

Tong, Y., A. Ben-Shlomo, C. Zhou, K. Wawrowsky, and S. Melmed. 2008. Pituitary tumor transforming gene 1 regulates Aurora kinase A activity. *Oncogene*. 27:6385–6395. http://dx.doi.org/10.1038/onc.2008.234

Zatsepina, O.V., N.Zh. Zhelev, and G.J. Jordan. 1995. [Immunolocalization of nucleolar protein B23 in mitotic centrosomes]. *Mol. Biol. (Mosk.)*. 29: 1359–1367.